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(54) Title: MODIFIED α-AMYLASES HAVING ALTERED CALCIUM BINDING PROPERTIES

(57) Abstract

Novel α-amylase enzymes are disclosed in which a new calcium binding site is modified by chemically or genetically altering residues associated with that calcium binding site. The novel α-amylases have altered performance characteristics, such as low pH starch hydrolysis performance, stability and activity profiles.

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MODIFIED α-AMYLASES HAVING ALTERED CALCIUM BINDING PROPERTIES

Field of the Invention

The present invention is directed to α-amylases having altered calcium binding properties. Particularly, the present invention is directed to novel α-amylase enzymes having modifications thereto, for example point mutations, which are intended to alter the binding of calcium at a previously unknown calcium binding site in the molecule. By altering the calcium binding properties at this additional site, it is possible to improve the stability of the modified α-amylase.

Background of the Invention

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) hydrolyze internal α-1,4-glucosidic linkages in starch, largely at random, to produce smaller molecular weight malto-dextrins. α-Amylases are of considerable commercial value, being used in the initial stages (liquefaction) of starch processing; in alcohol production; as cleaning agents in detergent matrices; and in the textile industry for starch desizing. α-Amylases are produced by a wide variety of microorganisms including *Bacillus* and *Aspergillus*, with most commercial amylases being produced from bacterial sources such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis* or *Bacillus stearothermophilus*. In recent years, the preferred enzymes in commercial use have been those from *Bacillus licheniformis* because of their heat stability and performance, at least at neutral and mildly alkaline pH's.

In U.S. Patent No. 5,322,778, liquefaction between pH 4.0 and 6.0 was achieved by adding an antioxidant such as bisulfite or a salt thereof, ascorbic acid or a salt thereof, erythorbic acid, or phenolic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene or a-tocopherol to the liquefaction slurry. According to this patent, sodium bisulfite must be added in a concentration of greater than 5 mM.

In U.S. Patent No. 5,180,669, liquefaction between a pH of 5.0 to 6.0 was achieved by the addition of carbonate ion in excess of the amount needed to buffer the solution to the ground starch slurry. Due to an increased pH effect which occurs with addition of carbonate ion, the slurry is generally neutralized by adding a source of hydrogen ion, for example, an inorganic acid such as hydrochloric acid or sulfuric acid.

In PCT Publication No. WO95/10603, α-amylase variants are disclosed which have improved laundry or dishwashing performance and comprise a mutation other than a single mutation at position M197 in *Bacillus licheniformis* α-amylase.

In PCT Publication No. WO94/02597, a mutant α-amylase having improved oxidative stability is described wherein one or more methionines are replaced by any amino acid except cysteine or methionine.

In PCT Publication No. WO94/18314, a mutant α-amylase having improved oxidative stability is described wherein one or more of the methionine, tryptophan, cysteine, histidine or tyrosine residues is replaced with a non-oxidizable amino acid.

In PCT Publication No. WO 91/00353, the performance characteristics and problems associated with liquefaction with wild-type *Bacillus licheniformis* α-amylase are approached by genetically engineering the α-amylase to include the specific substitutions Ala-111-Thr, His-133-Tyr and/or Thr-149-Ile.

Studies using recombinant DNA techniques to explore which residues are important for the catalytic activity of amylases and/or to explore the effect of modifying certain amino acids within the active site of various amylases and glycosylases have been conducted by various researchers (Vihinen et al., J. Biochem., vol. 107, pp. 267-272 (1990); Holm et al., Protein Engineering, vol. 3, pp. 181-191 (1990); Takase et al., Biochemica et Biophysica Acta, vol. 1120, pp. 281-288 (1992); Matsui et al., Febs Letters, vol. 310, pp. 216-218 (1992); Matsui et al., Biochemistry, vol. 33, pp. 451-458 (1992); Sogaard et al., J. Biol. Chem., vol. 268, pp. 22480-22484 (1993); Sogaard et al., Carbohydrate Polymers, vol. 21, pp. 137-146 (1993); Svensson, Plant Mol. Biol., vol. 25, pp. 141-157 (1994); Svensson et al., J. Biotech. vol. 29, pp. 1-37 (1993)). Researchers have also studied which residues are important for thermal stability (Suzuki et al., J. Biol. Chem., vol. 264, pp. 18933-18938 (1989); Watanabe et al., Eur. J. Biochem., vol. 226, pp. 277-283 (1994)); and one group has used such methods to introduce mutations at various histidine residues in a *Bacillus licheniformis* amylase, the rationale being that *Bacillus licheniformis* amylase, which is known to be relatively thermostable when compared to other similar *Bacillus* amylases, has an excess of histidines and, therefore, it was suggested that replacing a histidine could affect the thermostability of the enzyme. This work resulted in the identification of stabilizing mutations at the histidine residue at the +133 position and the alanine residue at position +209 (Declerck et al., J. Biol. Chem., vol. 265, pp. 15481-15488 (1990); FR 2 665 178-A1; Joyet et al., Bio/Technology, vol. 10, pp. 1579-1583 (1992)).

α-Amylases from different organisms have been shown to exhibit similar three-dimensional structure despite considerable differences in primary structure. Figure 1 illustrates the structure of α-amylase of *Bacillus licheniformis*. While some inter-species variation will exist between the various α-amylases, it is believed that the major structural elements of *Bacillus licheniformis* α-amylase are representative of α-amylase structures in general (see Brayer et al., Protein Sci., vol. 4, pp. 1730-1742 (1995); Larson et al., J. Mol. Biol., vol. 235, pp. 1560-1584 (1994); Qian et al., J. Mol. Biol., vol. 231, pp. 785-799 (1993)). For example, site-specific mutagenesis has identified three invariant carboxylates and two invariant histidines (D231, E261, D328 and H104 and H327 in *Bacillus licheniformis* α-

amylase), important for catalysis (Svensson, Plant Mol. Biol., vol. 25, p. 141 (1994)), and a general mechanism has been proposed (Mazur et al., Biochem. Biophys. Res. Comm., vol. 204, p. 297 (1994)). Residues found which are believed to be implicated in calcium and chloride binding have been characterized and found to be highly conserved among the different enzymes (see, e.g., Kadziola et al., J. Mol. Biol., vol. 239, p. 104 (1994); Qian et al., supra; Larson et al., supra; Brayer et al., supra; Machius et al., J. Mol. Biol., vol. 246, pp. 545-559 (1995); and Boel et al., Biochem., vol. 29, p. 6244 (1990)).

Moreover, homologies have been found between almost all endo-amylases sequenced to date, ranging from plants, mammals and bacteria (Nakajima et al., Appl. Microbiol. Biotechnol., vol. 23, pp. 355-360 (1986); Rogers, Biochem. Biophys. Res. Commun., vol. 128, pp. 470-476 (1985); Janecek, Eur. J. Biochem., vol. 224, pp. 519-524 (1994)). There are four areas of particularly high homology in certain *Bacillus* amylases, as shown in Figure 5, wherein the underlined sections designate the areas of high homology. Sequence alignments have also been used to map the relationship between *Bacillus* endo-amylases (Feng et al., J. Molec. Evol., vol. 35, pp. 351-360 (1987)). The relative sequence homology between *Bacillus stearothermophilus* and *Bacillus licheniformis* amylase is about 66% and that between *Bacillus licheniformis* and *Bacillus amyloliquefaciens* amylases is about 81%, as determined by Holm et al., Protein Engineering, vol. 3, no. 3, pp. 181-191 (1990). While sequence homology is important, it is generally recognized that structural homology is also important in comparing amylases or other enzymes.

Three dimensional structure similarities between various α-amylases (and related amylolytic enzymes like cyclodextrin glycosyltransferases and α-glucosidases) from different organisms, despite differences in their primary structure, are found in the common presence of an α/β-barrel forming a central part (domain A), a Greek key motif as a separate domain C and at least one additional domain, domain B (Machius et al., supra). Substrate binding is believed to be localized to a cleft between the α/β-barrel and domain B, comprising several β strands of variable length, depending on the species (Machius, supra). Also common is a requirement for calcium which is believed to maintain structural integrity. Machius discloses a calcium binding site implicating residues corresponding to N104, D200 and H235 derived from the crystal structure of a calcium depleted α-amylase from *Bacillus licheniformis*. In addition to the structure for *Bacillus licheniformis*, the structures for *Aspergillus niger* (Brady et al., Acta Crystallog. B, vol. 47, p. 527 (1991)), pig pancreas (Qian et al., J. Mol. Biol., vol. 231, p. 758 (1993); Larson et al., J. Mol. Biol., vol. 235, p. 1560 (1994)), and human pancreas (Brayer et al., Prot. Sci., vol. 4, p. 1730 (1995)) have been determined.

Despite the advances made in the prior art, a need exists for an α-amylase which has altered performance, including activity and stability, to facilitate their use in starch liquefaction,

detergents for laundry and dishwashing, baking, textile desizing and other standard uses for amylase. Because commercially available amylases are not acceptable under many conditions due to stability and/or activity problems, there is a need for an amylase having altered, and preferably increased, performance profiles under such conditions. For example, high alkalinity and oxidant (bleach) levels associated with detergents or the extreme conditions present during starch liquefaction can result in both destabilization and lack of activity from α-amylase. Thus, altered performance characteristics such as thermostability, pH stability, oxidative stability or calcium stability which can be achieved while also altering, maintaining, or increasing enzymatic activity as compared to the wild-type or precursor enzyme, would be desirable. Similarly, many α-amylases are known to require the addition of calcium ion for stability. This is undesirable in some applications due to increased processing costs.

Summary of the Invention

It is an object of the present invention to provide an α-amylase having altered performance profiles, e.g., altered pH stability, alkaline stability, oxidative stability, thermal stability or enzymatic activity.

It is a further object of the invention to provide an α-amylase having altered calcium binding properties, for example, having reduced need for added calcium to maintain activity levels.

It is a further object of the present invention to provide an α-amylase having improved performance due to increased low pH stability or activity, especially during liquefaction of starch.

It is still a further object of the present invention to provide an α-amylase having improved performance in high temperature or pH environments or in the presence of oxidants or bleach.

It is still a further object of the present invention to provide an α-amylase having improved performance in textile desizing or baking due to altered stability or activity.

According to the present invention, an α-amylase is provided comprising an A domain, a C domain and a calcium binding site, wherein the calcium binding site is associated with the A domain and the C domain and comprises ligand residues in the A domain and/or the C domain, wherein the α-amylase is modified to alter the characteristics of the calcium binding site and thereby alter the performance of the α-amylase.

In a preferred embodiment, the modification comprises a genetic modification resulting in a substitution, deletion or addition at a residue equivalent to one or more of amino acid residues 290-309, 339-347, 402-411, 426-436 or 472-477 in *Bacillus licheniformis* α-amylase. In an especially preferred embodiment, the genetic modification comprises substitution,

deletion or addition at a residue equivalent to one or more of G301, M304, H405, H406 and/or K436 in *Bacillus licheniformis* α-amylase.

In a composition embodiment, the present invention comprises a DNA which encodes the α-amylase of the invention. In a further composition embodiment, the present invention comprises an expression vector incorporating a DNA which encodes the α-amylase according to the invention, as well as a host cell into which such DNA and/or expression vector has been transformed. A method embodiment comprises expressing a DNA encoding the α-amylase of the invention or an expression vector incorporating such DNA in a host cell.

In a further composition embodiment, the present invention comprises a laundry or dishwashing detergent composition which incorporates the α-amylase according to the invention. In another composition embodiment, the present invention comprises a textile desizing composition which incorporates the α-amylase according to the invention. In yet another composition embodiment, the present invention comprises a starch liquefaction composition which incorporates the α-amylase according to the invention. In yet another composition embodiment, the present invention comprises a baking aid comprising the α-amylase according to the invention.

In a process embodiment of the present invention, a method of laundering clothing or washing dishes with a dishwashing detergent composition which incorporates the α-amylase according to the invention is provided. In another process embodiment of the present invention, a method of desizing textiles with a composition which incorporates the α-amylase according to the invention is provided. In yet another process embodiment of the present invention, a method of liquefying starch with a starch liquefaction composition which incorporates the α-amylase according to the invention is provided. In yet another process embodiment of the present invention, a method of baking is provided comprising adding a composition which incorporates the α-amylase according to the invention.

The modified α-amylases according to the present invention will provide several important advantages when compared to prior art α-amylases. For example, one advantage is found in variants having increased activity at low pH and high temperatures typical of common starch liquefaction methods. Another advantage is found in variants having increased high pH and oxidative stability which facilitates their use in detergents. Yet another advantage is provided by variants having improved stability in the absence or low concentration of calcium ion. The objects and attendant advantages of the present invention will be made more clear in the following detailed description and examples.

Brief Description of the Drawings

Figure 1 illustrates the structure of *Bacillus licheniformis* α-amylase showing the main-chain folding and the location of the calcium binding site associated with the A domain and

the B domain (CalA) and a second calcium binding site associated with the A domain and the C domain (CalB).

Figure 2 illustrates the stereo view of the final 2fo-fc difference map and the Sm anomalous difference Fourier at the calcium binding site associated with the A domain and the C domain of α-amylase derived from *Bacillus licheniformis*.

Figures 3A-C illustrate the DNA sequence of the gene for α-amylase from *Bacillus licheniformis* (NCIB 8061) and deduced amino acid sequence of the translation product as described by Gray et al., J. Bacteriology, vol. 166, pp. 635-643 (1986).

Figure 4 illustrates the amino acid sequence of the mature α-amylase enzyme from *Bacillus licheniformis*.

Figures 5A-B illustrate an alignment of the primary structures of three *Bacillus* α-amylases. The *Bacillus licheniformis* α-amylase (Am-Lich) is described by Gray et al., J. Bacteriology, vol. 166, pp. 635-643 (1986); the *Bacillus amyloliquefaciens* α-amylase (Am-Amylo) is described by Takkinen et al., J. Biol. Chem., vol. 258, pp. 1007-1013 (1983); and the *Bacillus stearothermophilus* α-amylase (Am-Stearo) is described by Ihara et al., J. Biochem., vol. 98, pp. 95-103 (1985).

Detailed Description of the Invention

"α-Amylase" means an enzymatic activity which cleaves or hydrolyzes the α(1-4) glycosidic bond, e.g., that in starch, amylopectin or amylose polymers. α-Amylase as used herein includes naturally occurring α-amylases as well as recombinant α-amylases. The α-amylases according to the present invention may be derived from a precursor amylase. The precursor α-amylase is produced by any source capable of producing α-amylase. Suitable sources of α-amylases are prokaryotic or eukaryotic organisms, including fungi, bacteria, plants or animals. Preferably, the precursor α-amylase is produced by a *Bacillus* or a fungus, for example those derived from *Aspergillus* (i.e., *A. oryzae* and *A. niger*). More preferably, the precursor is produced by *Bacillus licheniformis*, *Bacillus amyloliquefaciens* or *Bacillus stearothermophilus;* more preferably, the precursor α-amylase is derived from *Bacillus licheniformis*.

A "modified" α-amylase is an α-amylase which has been subjected to genetic or chemical modification so as to change its biochemical, structural or physico-chemical properties. A "genetic modification" in α-amylase means that the DNA sequence encoding a naturally occurring or precursor α-amylase has been modified to produce a mutant DNA sequence which encodes the substitution, insertion or deletion of one or more amino acids in the α-amylase sequence compared to the naturally occurring α-amylase or a precursor α-amylase.

"Expression vector" means a DNA construct comprising a DNA sequence which is capable of effecting the expression of said DNA in a suitable host, generally being operably linked to a suitable control sequence. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome-binding sites, and sequences which control termination of transcription and translation. A preferred promoter is the *Bacillus subtilis aprE* promoter. The vector may be a plasmid, a phage particle, or DNA intended to effect genomic insertion, i.e., integration. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. Plasmid and vector are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art, particularly including phage display.

"Host strain" or "host cell" means a suitable host for, e.g., an expression vector comprising DNA encoding the α-amylase according to the present invention. Host cells useful in the present invention are generally procaryotic or eucaryotic hosts, including any transformable microorganism in which the expression of α-amylase according to the present invention can be achieved. Specifically, host strains of the same species or genus from which the α-amylase is derived are suitable, such as a *Bacillus* strain. Preferably, an α-amylase negative *Bacillus* strain (genes deleted) and/or an α-amylase and protease deleted *Bacillus* strain (e.g., Δ*amyE*, Δ*apr*, Δ*npr*) is used. Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the α-amylase and its variants (mutants) or expressing the desired α-amylase.

"Liquefaction" or "liquefy" means a process by which starch is converted to shorter chain and less viscous dextrins. Generally, this process involves gelatinization of starch simultaneously with or followed by the addition of α-amylase.

"Calcium binding site" means a region within α-amylase which is suitable for and acts to bind a calcium ion in the presence of free calcium. Calcium is generally believed to be required to maintain the structural integrity of α-amylase under many conditions and the amino acid residues involved in calcium binding have been shown to be highly conserved among the different enzymes (Machius et al., J. Mol. Biol., vol. 246, pp. 545-559 (1995)). According to the present invention, the characteristics of the calcium binding site are altered compared to a wild-type or precursor α-amylase so as to alter the performance of the α-amylase. Alteration of the calcium binding site may include reducing or increasing the affinity of the site to bind calcium ion. By altering the performance is intended to mean the stability

(e.g., oxidative or thermal) or the activity (e.g., the rate or efficiency with which the α-amylase hydrolyzes starch substrate) of the enzyme in its various applications.

"Ligand residues" or "calcium ligand" means an amino acid residue or residues within an α-amylase enzyme which forms a ligand with calcium ion bound within a calcium binding site. With respect to the calcium binding site within α-amylase discovered by Applicants, five amino acid ligands have been identified which are believed to act as calcium ligands. The calcium ligand residues comprise amino acid residues equivalent to G300, Y302, H406, D407 and D430 in *Bacillus licheniformis* α-amylase. Specifically with respect to these identified calcium ligands, the carbonyl oxygens of G300, Y302 and H406 and the side-chains of D407 and D430 are believed to be implicated in binding calcium.

According to the present invention, an α-amylase comprising an A domain, a C domain, and a calcium binding site is provided, wherein the calcium binding site is associated with the A domain and the C domain and comprises ligand residues in the A domain and/or the C domain, wherein the α-amylase is modified to alter the characteristics of the calcium binding site and thereby alter the performance of the α-amylase.

Also provided is a nucleic acid molecule (DNA) which encodes an amino acid sequence comprising at least a part of the α-amylase provided by the present invention, expression systems incorporating such DNA including vectors and phages, host cells transformed with such DNA, and anti-sense strands of DNA corresponding to the DNA molecule which encodes the amino acid sequence. Similarly, the present invention includes a method for producing an α-amylase by expressing the DNA incorporated on an expression system which has been transformed into a host cell.

The DNA sequences may be expressed by operably linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate host according to well known techniques. A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, include segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as the various known plasmids and phages useful for this purpose. In addition, any of a wide variety of expression control sequences are generally used in these vectors. For example, Applicants have discovered that a preferred expression control sequence for *Bacillus* transformants is the *aprE* signal peptide derived from *Bacillus subtilis*. Additionally, phage display systems are useful for the invention herein.

A wide variety of host cells are also useful in expressing the DNA sequences of this invention and are contemplated herein. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli, Pseudomonas, Bacillus, Streptomyces*, various

fungi, e.g., *Trichoderma* or *Aspergillus*, yeast and animal cells. Preferably, the host expresses the α-amylase of the present invention extracellularly to facilitate purification and downstream processing. Expression and purification of the mutant α-amylase of the invention may be effected through art-recognized means for carrying out such processes.

The α-amylases according to the present invention comprise an amino acid sequence which is derived from the amino acid sequence of a precursor α-amylase. The precursor α-amylases include naturally occurring α-amylases and recombinant α-amylases. The amino acid sequence of the α-amylase mutant is derived from the precursor α-amylase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is generally of the precursor DNA sequence which encodes the amino acid sequence of the precursor α-amylase rather than manipulation of the precursor α-amylase enzyme *per se*. Methods for modifying α-amylase genes (i.e., through site-directed oligonucleotide mutagenesis) and transforming, expressing and secreting enzyme products produced pursuant to the mutagenized gene have been described in the prior art, including PCT Publication No. WO95/10603 (Novo Nordisk), PCT Publication No. WO94/02597 (Novo Nordisk), PCT Publication No. WO94/18314 (Genencor International, Inc.) and PCT Publication No. WO91/00353 (Gist Brocades), such disclosures being incorporated by reference. Additional suitable methods for manipulation of the precursor DNA sequence include methods disclosed herein and in commonly owned U.S. Patent Nos. 4,760,025 and 5,185,258, incorporated herein by reference.

The major structural elements, including the newly discovered CalB site which is disclosed herein, and changes thereto to alter the performance of an α-amylase are described below in general terms as applicable to most α-amylases. As shown in Figure 1, three major domains are defined, the A domain, the B domain and the C domain, as well as two calcium binding sites, CalA and CalB. The A domain comprises the central portion of the molecule and has been identified as an α/β or TIM barrel. The α/β barrel is made of a series of parallel β-strands which are interconnected by α-helices. On the carboxyl end of the enzyme on one side of the A domain is a region comprising an anti-parallel β-barrel known as a "Greek key" motif (see, e.g., Richardson et al., Advan. Protein Chem., vol. 34, 167-339 (1981); Braden et al., Introduction to Protein Structure, Garland Publishing Inc., New York (1991)). This domain has been identified as the C domain. On the opposite side of the A domain from the C domain (the N-terminal) is an additional domain which comprises several β strands of variable length depending on the species, known as the B domain. The B domain has been recognized as being highly variable between α-amylases of different species and often comprises extended loops. It is believed that substrate binding is localized to a cleft between the A domain and the B domain and that the active site is further associated with this

region of the molecule. The CalA binding site is located within a cleft separating the A domain and the B domain and is believed to provide stability to this region. The CalB binding site disclosed herein is located in the region where the A domain and the C domain interface.

The discovery of the CalB binding site in a *Bacillus* α-amylase by Applicants has enabled Applicants to develop mutant α-amylases having altered performance, and particularly altered stability. For example, general principles for stabilization of protein structure may be applied to the region around the CalB site. Additionally, strategies specifically designed to improve calcium binding at the CalB site may be implemented to increase the stability of the enzyme. Preferably, such modifications are within 15 angstroms of the center of mass of the calcium bound to the CalB binding site, more preferably within 10 angstroms of the center of mass of the calcium bound to the CalB binding site.

Residues in α-amylase are identified herein for deletion or substitution. Thus, specific residues discussed herein refer to an amino acid position number which references the number assigned to the mature *Bacillus licheniformis* α-amylase sequence illustrated in Figure 4. The invention, however, is not limited to the mutation of the particular mature α-amylase of *Bacillus licheniformis* but extends to non-*Bacillus licheniformis* precursor α-amylases containing amino acid residues at positions which are equivalent to the particular identified residue in *Bacillus licheniformis* α-amylase. A residue of a precursor α-amylase is equivalent to a residue of *Bacillus licheniformis* α-amylase if it is either homologous (i.e., corresponds in position for either the primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus licheniformis* α-amylase (i.e., having the same or similar functional capacity to combine, react, or interact chemically or structurally).

In order to establish homology to primary structure, the amino acid sequence of a precursor α-amylase is directly compared to the *Bacillus licheniformis* α-amylase primary sequence and particularly to a set of residues known to be invariant to all α-amylases for which sequences are known (see, e.g., Figure 7). It is possible also to determine equivalent residues by tertiary structure analysis of the crystal structures reported for porcine pancreatic α-amylase (Buisson et al., EMBO Journal, vol. 6, pp. 3909-3916 (1987); Qian et al., Biochemistry, vol. 33, pp. 6284-6294 (1994); Larson et al., J. Mol. Biol., vol. 235, pp. 1560-1584 (1994)); Taka-amylase A from *Aspergillus oryzae* (Matsuura et al., J. Biochem. (Tokyo), vol. 95, pp. 697-702 (1984)); and an acid α-amylase from *A. niger* (Boel et al.. Biochemistry, vol. 29, pp. 6244-6249 (1990)), with the former two structures being similar, and for barley α-amylase (Vallee et al., J. Mol. Biol., vol. 236, pp. 368-371(1994); Kadziola, J. Mol. Biol., vol. 239, pp. 104-121 (1994)). Although there have been some preliminary studies published (Suzuki et al., J. Biochem., vol. 108, pp. 379-381 (1990); Lee et al., Arch. Biochem. Biophys, vol. 291, pp. 255-257 (1991); Chang et al., J. Mol. Biol., vol. 229, pp. 235-238 (1993); Mizuno

et al., J. Mol. Biol., vol. 234, pp. 1282-1283 (1993)), there is only a published structure for *Bacillus licheniformis* α-amylase (Machius et al., J. Mol. Biol. vol. 246, pp. 545-549 (1995)). However, several researchers have predicted common super-secondary structures between glucanases (MacGregor et al., Biochem. J., vol. 259, pp. 145-152 (1989)) and within α-amylases and other starch-metabolizing enzymes (Jaspersen, J. Prot. Chem. vol. 12, pp. 791-805 (1993); MacGregor, Starke, vol. 45, pp. 232-237 (1993)); and sequence similarities between enzymes with similar super-secondary structures to α-amylases (Janecek, FEBS Letters, vol. 316, pp. 23-26 (1993); Janecek et al., J. Prot. Chem., vol. 12, pp. 509-514 (1993)). A structure for the *Bacillus stearothermophilus* enzyme has been modeled on that of Taka-amylase A (Holm et al., Protein Engineering, vol. 3, pp. 181-191 (1990)). The four highly conserved regions shown in Figure 7 contain many residues thought to be part of the active-site (Matsuura et al., J. Biochem. (Tokyo), vol. 95, pp. 697-702 (1984); Buisson et al., EMBO Journal, vol. 6, pp. 3909-3916 (1987); Vihinen et al., J. Biochem., vol. 107, pp. 267-272 (1990)) including His +105; Arg +229; Asp +231; His +235; Glu +261 and Asp +328 under the *Bacillus licheniformis* numbering system.

The segments of the α-amylase polypeptide chain which comprise the CaIB binding site include residues 290-309, 339-347, 402-411, 426-436 and 472-477. These polypeptide segments comprise the CaIB binding site. Accordingly, regiospecific random mutations in these regions would be expected to yield variants that modulate the stability of α-amylase via modulation of the affinity of calcium at this site.

Additional more specific strategies are provided below:

(1) Increasing the entropy of main-chain unfolding may introduce stability to the enzyme. For example, the introduction of proline residues into position 2 of reverse turns at the N-termini of α-helices and in loop structures may significantly stabilize the protein by increasing the entropy of the unfolding (see, e.g., Watanabe et al., Eur. J. Biochem., vol. 226, pp. 277-283 (1994)). Similarly, glycine residues have no β-carbon, and thus have considerably greater backbone conformational freedom than many other residues. This may lead to high flexibility with resultant weak stability. Replacement of glycines at one or more residues equivalent to G299, G410, G433, G474, G475 in *Bacillus licheniformis*, preferably with an alanine, may reduce the flexibility and improve stability. Additionally, by shortening external loops it may be possible to improve stability. It has been observed that hyperthermophile produced proteins have shorter external loops than their mesophilic homologues (see, e.g., Russel et al., Current Opinions in Biotechnology, vol. 6, pp. 370-374 (1995). The introduction of disulfide bonds may also be effective to stabilize distinct tertiary structures in relation to each other. Modification at G301 would alter the stability of the segment at 290-309 by restricting or improving the conformational variability of glycine.

Specifically contemplated are substitutions of aspartic acid or proline at this residue. Modification at G474 by replacement with another residue may increase stability by introducing a Cβ, thus lowering its conformational freedom.

(2) Decreasing internal cavities by increasing side-chain hydrophobicity may alter the stability of an enzyme. Reducing the number and volume of internal cavities increases the stability of enzyme by maximizing hydrophobic interactions and reducing packing defects (see, e.g., Matthews, Ann. Rev. Biochem., vol. 62, pp. 139-160 (1993); Burley et al., Science, vol. 229, pp. 23-29 (1985); Zuber, Biophys. Chem., vol. 29, pp. 171-179 (1988); Kellis et al., Nature, vol. 333, pp. 784-786 (1988)). It is known that multimeric proteins from thermophiles often have more hydrophobic sub-unit interfaces with greater surface complementarity than their mesophilic counterparts (Russel et al., supra). This principle is believed by Applicants to be applicable to domain interfaces of monomeric proteins. Specific substitutions that may improve stability by increasing hydrophobicity include lysine to arginine, serine to alanine and threonine to alanine (Russel et al., supra). Modification at G301 by substitution to alanine or proline may increase side-chain size with resultant reduction in cavities, better packing and increased hydrophobicity. Additionally, a cavity at the interface between domain A and domain C in the CalB binding region is bordered by Y302, M304, L307, F343, L427 and I428. Substitutions to reduce the size of the cavity, increase hydrophobicity and improve the complementarity of the A domain-C domain interface may improve stability of the enzyme. Specifically, modification of the specific residue at these positions with a different residue selected from any of phenylalanine, tryptophan, tyrosine, leucine and isoleucine may improve performance. Additional substitutions which may be useful are at V409 and F403, preferably the substitutions at V409 comprise isoleucine or leucine, and at F403 comprise tyrosine or tryptophan.

(3) Balancing charge in rigid secondary structure, i.e., α-helices and β-turns may improve stability. For example, neutralizing partial positive charges on a helix N-terminus with negative charge on aspartic acid may improve stability of the structure (see, e.g., Eriksson et al., Science, vol. 255, pp. 178-183 (1992)). Similarly, neutralizing partial negative charges on helix C-terminus with positive charge may improve stability. Removing positive charge from interacting with peptide N-terminus in β-turns should be effective in conferring tertiary structure stability. Substitution of H405 with a non-positively charged residue could remove an unfavorable positive charge from interacting with the amide nitrogen of D407 in the 405-408 turn.

(4) Introducing salt bridges and hydrogen bonds to stabilize tertiary structures may be effective. For example, ion pair interactions, e.g., between aspartic acid or glutamic acid and lysine, arginine or histidine, may introduce strong stabilizing effects and may be used to attach different tertiary structure elements with a resultant improvement in thermostability.

Additionally, increases in the number of charged residue/non-charged residue hydrogen bonds, and the number of hydrogen-bonds generally, may improve thermostability (see, e.g., Tanner et al., Biochemistry, vol. 35, pp. 2597-2609). Substitution of H405 with aspartic acid, asparagine, glutamic acid or glutamine may introduce a hydrogen bond with the backbone amide of D407, thus stabilizing the 405-408 turn. Substitution at K436 with arginine may improve the salt bridge with D404 and introduce an H-bond into the backbone carbonyl of I408.

(5) Avoiding thermolabile residues in general may increase thermal stability. For example, asparagine and glutamine are susceptible to deamidation and cysteine is susceptible to oxidation at high temperature. Reducing the number of these residues in sensitive positions may result in improved thermostability (Russel et al., supra). Substitution or deletion at Q291, Q298, N309, Q340 or N473 by any residue other than glutamine or cysteine may increase stability by avoidance of a thermolabile residue.

(6) Introducing a sixth ligand from the protein to the calcium ion may improve the stability of the bound calcium and, thus, the enzyme. Substitution of H406 with aspartic acid, asparagine, glutamic acid or glutamine may increase the calcium affinity.

(7) Stabilization of the existing ligands to calcium in CalB may also improve stability of the bound calcium and, thus, the enzyme. For example, M304 may be substituted with phenylalanine or tyrosine to introduce aromatic side-chain/aspartic acid side-chain stabilization where the carboxylate oxygen may interact favorably with the partial positive charge associated with benzyl rings, increasing the stability of D407 and D430. Substitution of H405 with phenylalanine or tyrosine to introduce a hydrophobic group near D407, may increase the stability of D407 via formation of favorable van der Waals interactions with C-b and C-g atoms of the D407 side-chain. Substitution at G300 with phenylalanine may remove the side-chain H-bond to Q291.

(8) Increasing the electronegativity of any of the calcium ligands may improve calcium binding. For example, substitution of M304 with phenylalanine or tyrosine may increase the electronegativity of D407 and D430 by improved shielding from solvent, thereby improving calcium binding.

(9) Removing positive-charges in the vicinity of the calcium ion that may interfere with the calcium binding should similarly improve the calcium binding site stability. For example, substituting H405 or H406, which are in the immediate vicinity of the bound calcium, may have positive charge that could produce unfavorable charge-charge interactions with the positively charged calcium ion and may have competing charge-charge interactions with negatively charged calcium ligands. Thus, replacement with a suitable non-positively charged residue may increase calcium affinity and protein stability.

(10) Stabilization of the CalB binding site by introducing negatively charged residues in the vicinity thereof may also improve the binding of the calcium ion in the site (see, e.g., Pantoliano et al., Biochemistry, vol. 27, pp. 8311-8317 (1988); Bryan, Stability of Protein Pharmaceuticals Part B: In vitro Pathways for Degradation and Strategies for Protein Stabilization (Ahern & Manning, Eds.), pp. 147-181 (1992); Fagain, Biochim. Biophys. Acta, vol. 1252, pp. 1-14 (1995)). For example, substitution of Q291, Q298, N309, Q304, H405, H406, N473 and/or G474 with negatively charged aspartic acid or glutamic acid will increase the net negative charge in the calcium area and may increase calcium affinity and, thus, enzyme stability.

The α-amylases according to the present invention may exhibit altered performance characteristics providing desirable and unexpected results which are useful in the various applications for which α-amylases are commonly used. For example, α-amylases according to the present invention which exhibit altered performance characteristics at low pH, including improved thermostability, improved pH stability and/or improved oxidative stability, are useful in low pH liquefaction of starch. Enhanced thermostability will be useful in extending the shelf life of products which incorporate them. Enhanced oxidative stability or improved performance is particularly desirable in cleaning products, and for extending the shelf life of α-amylase in the presence of bleach, perborate, percarbonate or peracids used in such cleaning products. To the contrary, reduced thermal stability or oxidative stability may be useful in industrial processes which require the rapid and efficient quenching of amylolytic activity. Additionally, a reduced requirement or stronger affinity for calcium would be advantageous in the presence of sequestering components generally found in detergents, i.e., builders.

The α-amylase of the present invention is especially useful in starch processing and particularly in starch liquefaction. Conditions present during commercially desirable liquefaction processes characteristically include low pH, high temperature and potential oxidation conditions requiring α-amylases exhibiting improved low pH performance, improved thermal stability and improved oxidative stability. Accordingly, α-amylases according to the present invention which are particularly useful in liquefaction exhibit improved performance at a pH of less than about 6, preferably less than about 5.5, and more preferably between about 5.0 and 5.5. Additionally, α-amylases according to the present invention which exhibit increased thermal stability at temperatures of between about 80-120°C, and preferably between about 100-110°C, and increased stability in the presence of oxidants will be particularly useful. Preferably, the α-amylase according to the present invention which is used in liquefaction further comprises a deletion or substitution at one or more of positions M15, V128, H133, W138, N188, A209 and/or M197.

In another embodiment of the present invention there are provided detergent compositions in either liquid, gel or granular form, which comprise the α-amylase according to the present invention. Such detergent compositions will particularly benefit from the addition of an α-amylase according to the present invention which has increased thermal stability to improve shelf-life or increased oxidative stability such that the α-amylase has improved resistance to bleach or peracid compounds commonly present in detergents. Thus, α-amylase according to the present invention may be advantageously formulated into known powdered, liquid or gel detergents having a pH of between about 6.5 and about 12.0. A preferred embodiment of the present invention further comprises a deletion or substitution at one or more of positions M15, V128, H133, W138, N188, A209 and/or M197. Detergent compositions comprising the α-amylase according to the present invention may further include other enzymes such as endoglycosidases, cellulases, proteases, lipases or other amylase enzymes, for example amylase derived from *Bacillus stearothermophilus*, as is generally known in the art.

Embodiments of the present invention which comprise a combination of the α-amylase according to the present invention with protease enzymes preferably include oxidatively stable proteases such as those described in U.S. Re 34,606, incorporated herein by reference, as well as commercially available enzymes such as DURAZYM (Novo Nordisk), MAXAPEM (Gist-brocades) and PURAFECT® OxP (Genencor International, Inc.). Methods for making such protease mutants (oxidatively stable proteases), and particularly such mutants having a substitution for the methionine at a position equivalent to M222 in *Bacillus amyloliquefaciens*, are described in U.S. Re 34,606.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims. Abbreviations used herein, particularly three letter or one letter notations for amino acids are described in Dale, J.W., Molecular Genetics of Bacteria, John Wiley & Sons, (1989) Appendix B.

Experimental

Example

Preparation of *Bacillus licheniformis* α-Amylase Crystals

Crystals were grown in 10μL hanging drops, from 1.6-1.8 M Li_2SO_4, 1 mM $CaCl_2$, 50 mM NaCl, buffered at pH 6.5 with 200 mM bistrispropane. The crystals grow as elongated prisms, to a maximum dimension of approximately 1.5 mm, in 7-14 days. The space group is $P2_12_12_1$, with a = 118.3 Å, b = 119.0 Å and c = 84.9 Å. The Matthews No. (see Matthews, J., Mol. Biol., vol. 33, pp. 409 (1968)) is 3.01, assuming 2 molecules in the asymmetric unit, which is within the normal range. Data were recorded using an RAXISII image plate system, mounted on an RU-200B rotating anode X-generator, producing graphite monochromated

CuK_α radiation. Data were processed and reduced to amplitudes using software distributed with the system by Molecular Structures Corp. (The Woodlands, Texas). The phase information was determined using multiple isomorphous replacement (MIR) supplemented with anomalous scattering data (MIRAS), and subsequent density modification. Heavy-atom derivatives were prepared by conventional soaking methods, except for the $SmCl_3$, derivative, which was prepared by co-crystallization. Heavy-atom positions were located using difference Pattersons and cross-phased difference Fouriers. Excellent anomalous scattering data was obtained for a $SmCl_3$ derivative, which was used to find the correct hand, and put all heavy-atoms on a common origin. Heavy-atom positions were refined and MIRAS phases calculated, using Xheavy (Zhang et al., Acta Crystallog. A, vol. 46, pp. 377 (1990)). Phases were improved by solvent flattening, with SQUASH (McRee, J., Mol. Graph., vol. 10, pp. 44 (1992)), resulting in a 3.0 Å map in which most of the secondary structure elements of both molecules could be identified. Model building, real space refinement and symmetry averaging were performed using Xfit (Zhang, supra). The Cα positions of the β-strands and α-helices of the α/β barrel domain, and the C-terminal of all β domains of both molecules were identified. The TIM barrel of *Aspergillus* α-amylase (PDB entry 6TAA,) (Swift et al., Acta Crystallog. B, vol. 47, pp. 535 (1991)) was approximately overlaid the Cα trace of both molecules of the asymmetric unit and were accurately positioned using real-space refinement of the entire unmodified domain. This allowed accurate determination of the local symmetry operator, which was used for non-crystallographic symmetry averaging of the map. This resulted in a significant improvement of the map, except for domain B. At this point, only one molecule was built to the symmetry averaged map, the second being generated using the local symmetry operator. The Cα positions were identified, and the main-chain built using overlapping pentamers, drawn from a database of well refined structures (Zhang, supra; Jones et al., EMBO, vol. 5, pp. 819 (1986)). At domain B, much of the map was uninterpretable and only residues 105-116 and 133-169 could be built. Dummy alanines were built for those residues for which no side-chain density was evident. This initial model was refined using a simulated annealing slowcool protocol (initial temp = 3000 K), followed by conventional least-squares refinement, using Xplor (Brunger et al., Acta Crystallog. A, vol. 45, p. 50 (1989)) for data between 15-3.0 Å ($F \geq 3\sigma$), with non-crystallographic symmetry restraints applied. This model converged at an R-factor of 0.28. MIRAS and model phases were combined using sigmaA (Read, Acta Crystallog. A, vol. 42, pp. 140 (1986)), to produce a 2.2 Å map. Missing residues were built, along with considerable manual adjustment of the rest of the structure, and then refined using simulated annealing (initial temp = 1000 K), using data between 8.0 and 2.2 Å ($F \geq 3\sigma$). The model converged at an R-factor of 0.245. Subsequent restrained isotropic B-factor refinement gave an R-factor of 0.225. SigmaA weighted 2fo-fc

and fo-fc maps were computed using calculated phases and used to identify errors, and to locate the calcium ions. Upon obtaining 1.9 Å native data, fo-fc and 2fo-fc difference maps were used to locate remaining errors and identify ordered water molecules, followed by Powell minimization and stereochemically restrained B-factor refinement.

The R-factor of the present model is 0.19, (15-1.9 Å, F≥3σF). The model contains 7914 non-hydrogen atoms, and includes 630 water oxygen atoms, and three calcium atoms. It shows good geometry, with r.m.s deviations of 0.012 Å and 1.35° from ideal bond lengths and angles, respectively. The Ramachandron plot of ϕ and φ angles shows that residue 150 is the only non-glycine residue to deviate significantly from allowed regions.

B. licheniformis α-amylase contains 483 residues. In the present model the first three residues of the N-terminus and the C-terminal residue are missing. Also missing are residues 181-195 of molecule 1, and 181-193 of molecule 2. The data derived from this example is provided in Table 1.

Table 1

Data set	Resolution	Rmerge	R-deriv.	N sites	Phasing power	Anom. Scatt.
Native	50-1.8 Å	0.09	-	-	-	-
$SmCl_3$	50-2.2 Å		0.073	4	1.45	Y
PtI_6	50-3.0 Å		0.259	5	1.22	N
$PtCl_4$	50-3.0 Å		0.249	5	1.29	N
$Hg(Ac)_2$	50-3.0 Å		0.124	4	1.33	N
$IrCl_6$	50-3.0 Å		0.226	4	1.01	N
HgI_3	50-2.2 Å		0.133	12[1]	1.48	Y
Me_3PbI	50-2.2 Å		0.186	2	1.29	Y

Claims

1. An α-amylase comprising an A domain, a C domain and a calcium binding site, wherein said calcium binding site is associated with said A domain and said C domain comprises ligand residues in said A domain and/or said C domain, wherein said α-amylase is modified to alter the characteristics of said calcium binding site and thereby alter the performance of said α-amylase.

2. The α-amylase according to Claim 1, wherein said modification to said α-amylase comprises genetic modification to substitute, add or delete one or more amino acid residues in said α-amylase.

3. The α-amylase according to Claim 2, wherein said α-amylase is produced by *Bacillus* or a derivative thereof.

4. The α-amylase according to Claim 3, wherein said α-amylase is produced by *Bacillus licheniformis, Bacillus amyloliquefaciens* or *Bacillus stearothermophilus* or a derivative thereof.

5. The α-amylase according to Claim 2, wherein said genetic modification comprises substituting, adding or deleting an amino acid residue at a position equivalent to positions 290-309, 339-347, 402-411, 426-436 and/or 472-477 in *Bacillus licheniformis*.

6. The α-amylase according to Claim 5, wherein said genetic modification comprises a substitution at one or more of Q291, Q298, G299, G301, Y302, M304, L307, N309, Q340, F343, F403, H405, H406, D407, V409, G410, L427, I428, D430, G433, K436, N473, G474 and G475 in *Bacillus licheniformis*.

7. A detergent comprising the α-amylase according to Claim 1.

8. A starch liquefaction composition comprising the α-amylase according to Claim 1.

9. The α-amylase according to Claim 1, wherein said α-amylase further comprises a substitution or deletion at one or more residues equivalent to M15, V128, H133, W138, N188, A209 and/or M197 in *Bacillus licheniformis*.

10. A method of liquefying starch comprising the step of adding an α-amylase according to Claim 1 to an aqueous solution of starch and incubating for a suitable time and under suitable conditions to liquefy the aqueous solution of starch.

11. A DNA encoding the α-amylase according to Claim 1.

12. An expression vector in which the DNA of Claim 11 is operably linked to an expression control sequence which may be employed in expressing said DNA.

13. A host cell transformed with the expression vector of Claim 12.

14. A method of expressing and/or secreting the DNA of Claim 11 in a host cell.

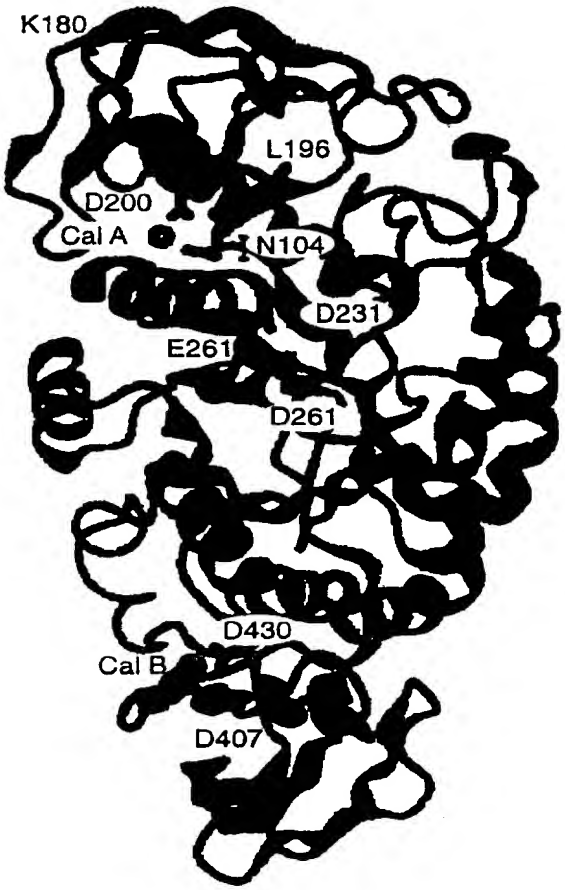

FIG._1

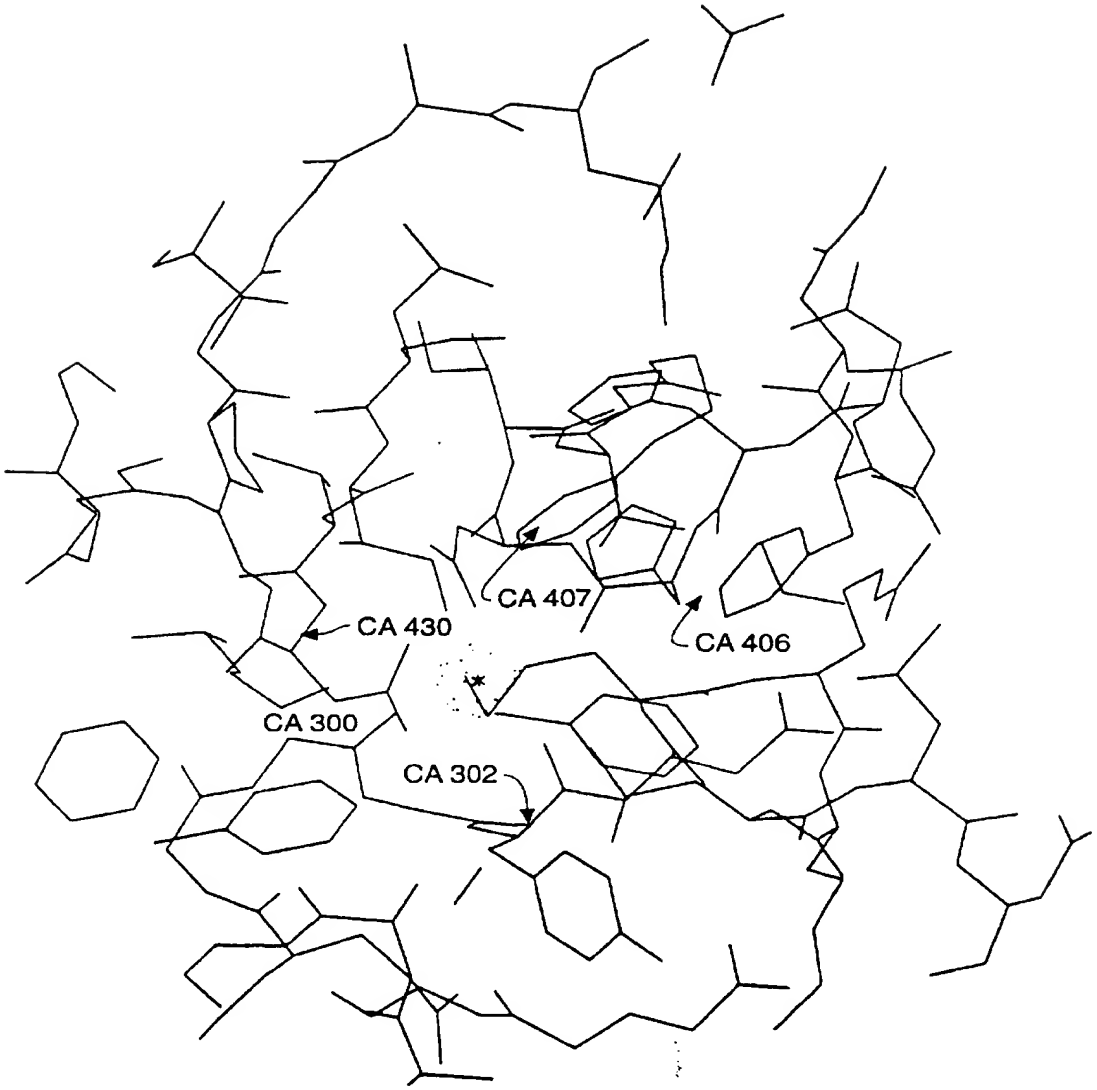

FIG._2

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          10                  30                  50
AGCTTGAAGAAGTGAAGAAGCAGAGAGGCTATTGAATAAATGAGTAGAAAGCGCCATATC

          70                  90                  110
GGCGCTTTTCTTTTGGAAGAAAATATAGGGAAAATGGTACTTGTTAAAAATTCGGAATAT

          130                 150                 170
TTATACAACATCATATGTTTCACATTGAAAGGGGAGGAGAATCATGAAACAACAAAAACG
                                                M  K  Q  Q  K  R

          190                 210                 230
GCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTCTTGCTGCCTCATTCTGC
 L  Y  A  R  L  L  T  L  L  F  A  L  I  F  L  L  P  H  S  A

          250                 270                 290
AGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAA
 A  A  A  A  N  L  N  G  T  L  M  Q  Y  F  E  W  Y  M  P  N

          310                 330                 350
TGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTAT
 D  G  Q  H  W  K  R  L  Q  N  D  S  A  Y  L  A  E  H  G  I

          370                 390                 410
TACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGG
 T  A  V  W  I  P  P  A  Y  K  G  T  S  Q  A  D  V  G  Y  G

          430                 450                 470
TGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTA
 A  Y  D  L  Y  D  L  G  E  F  H  Q  K  G  T  V  R  T  K  Y

          490                 510                 530
CGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGT
 G  T  K  G  E  L  Q  S  A  I  K  S  L  H  S  R  D  I  N  V

          550                 570                 590
TTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGC
 Y  G  D  V  V  I  N  H  K  G  G  A  D  A  T  E  D  V  T  A

          610                 630                 650
GGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGC
 V  E  V  D  P  A  D  R  N  R  V  I  S  G  E  H  L  I  K  A

          670                 690                 710
CTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTG
 W  T  H  F  H  F  P  G  R  G  S  T  Y  S  D  F  K  W  H  W

          730                 750                 770
GTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTT
 Y  H  F  D  G  T  D  W  D  E  S  R  K  L  N  R  I  Y  K  F

          790                 810                 830
TCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGAT
 Q  G  K  A  W  D  W  E  V  S  N  E  N  G  N  Y  D  Y  L  M
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FIG._3A

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          850                 870                 890
GTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCAC
 Y  A  D  I  D  Y  D  H  P  D  V  A  A  E  I  K  R  W  G  T

          910                 930                 950
TTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAA
 W  Y  A  N  E  L  Q  L  D  G  F  R  L  D  A  V  K  H  I  K

          970                 990                1010
ATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTT
 F  S  F  L  R  D  W  V  N  H  V  R  E  K  T  G  K  E  M  F

         1030                1050                1070
TACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAAC
 T  V  A  E  Y  W  Q  N  D  L  G  A  L  E  N  Y  L  N  K  T

         1090                1110                1130
AAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGAC
 N  F  N  H  S  V  F  D  V  P  L  H  Y  Q  F  H  A  A  S  T

         1150                1170                1190
ACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCC
 Q  G  G  G  Y  D  M  R  K  L  L  N  G  T  V  V  S  K  H  P

         1210                1230                1250
GTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTC
 L  K  S  V  T  F  V  D  N  H  D  T  Q  P  G  Q  S  L  E  S

         1270                1290                1310
GACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGG
 T  V  Q  T  W  F  K  P  L  A  Y  A  F  I  L  T  R  E  S  G

         1330                1350                1370
ATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAAT
 Y  P  Q  V  F  Y  G  D  M  Y  G  T  K  G  D  S  Q  R  E  I

         1390                1410                1430
TCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGG
 P  A  L  K  H  K  I  E  P  I  L  K  A  R  K  Q  Y  A  Y  G

         1450                1470                1490
AGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAG
 A  Q  H  D  Y  F  D  H  H  D  I  V  G  W  T  R  E  G  D  S

         1510                1530                1550
CTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCG
 S  V  A  N  S  G  L  A  A  L  I  T  D  G  P  G  G  A  K  R

         1570                1590                1610
AATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTC
 M  Y  V  G  R  Q  N  A  G  E  T  W  H  D  I  T  G  N  R  S

         1630                1650                1670
GGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGT
 E  P  V  V  I  N  S  E  G  W  G  E  F  H  V  N  G  G  S  V
```

FIG._3B

```
        1690                    1710                    1730
TTCAATTTATGTTCAAAGATAGAAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCCGTT
 S   I   Y   V   Q   R   *
        1750                    1770                    1790
TTTTTATTTTGCCCGTCTTATAAATTTCTTTGATTACATTTTATAATTAATTTTAACAAA

        1810                    1830                    1850
GTGTCATCAGCCCTCAGGAAGGACTTGCTGACAGTTTGAATCGCATAGGTAAGGCGGGGA

        1870                    1890                    1910
TGAAATGGCAACGTTATCTGATGTAGCAAAGAAAGCAAATGTGTCGAAAATGACGGTATC

        1930                    1950
GCGGGTGATCAATCATCCTGAGACTGTGACGGATGAATTGAAAAAGCT
```

FIG._3C

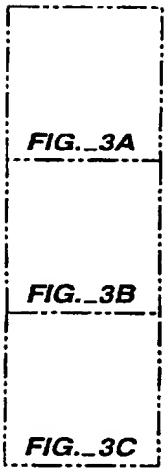


FIG._3

10 30 50
ANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD

70 90 110
LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEV

130 150 170
DPADRNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGK

190 210 230
AWDWEVSNENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSF

250 270 290
LRDWVNHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGG

310 330 350
GYDMRKLLNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQTWFKPLAYAFILTRESGYPQ

370 390 410
VFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWTREGDSSVA

430 450 470
NSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIY

VQR

FIG._4

Am-Lich = *B.Licheniformis* Am-Amylo = *B.amyloliquefaciens* Am-Stearo = *B.stearothermophilus*

```
                                                                   1           19
            1                                                                  60
Am-Lich     ..............MKQQ KRLYARLLTL LFALIFLLPH ...............SAAA AANLNGTLMQ YFEWYMPNDG
Am-Amylo    MRGRGNMIQK RKRTVSFRLV LMCTLLFVSL .................PITK TSAVNGTLMQ YFEWYTPNDG
Am-Stearo   ................VLTF HRIIRKGWMF LLAFLLTASL FCPTGRHAKA AAPFNGTMMQ YFEWYLPDDG

                                                                               79
            61                                                                 120
Am-Lich     QHWKRLQNDS AYLAEHGITA VWIPPAYKGT SQADVGYGAY DLYDLGEFHQ KGTVRTKYGT
Am-Amylo    QHWKRLQNDA EHLSDIGITA VWIPPAYKGL SQSDNGYGPY DLYDLGEFQQ KGTVRTKYGT
Am-Stearo   TLWTKVANEA NNLSSLGITA LSLPPAYKGT SRSDVGYGVY DLYDLGEFNQ KGTVRTKYGT

                                                                               139
            121                                                                180
Am-Lich     KGELQSAIKS LHSRDINVYG DVVINHKGGA DATEDVTAVE VDPADRNRVI SGEHLIKAWT
Am-Amylo    KSELQDAIGS LHSRNVQVYG DVVLNHKAGA DATEDVTAVE VNPANRNQET SEEYQIKAWT
Am-Stearo   KAQYLQAIQA AHAAGMQVYA DVVFDHKGGA DGTEWVDAVE VNPSDRNQEI SGTYQIQAWT

                                                                               197
            181                                                                240
Am-Lich     HFHFPGRGST YSDFKWHWYH FDGTDWDESR KLNRIYKF.... QGKAWDWEVS NENGNYDYLM
Am-Amylo    DFRFPGRGNT YSDFKWHWYH FDGADWDESR KISRIFKFRG EGKAWDWEVS SENGNYDYLM
Am-Stearo   KFDFPGRGNT YSSFKWRWYH FDGVDWDESR KLSRIYKFRG IGKAWDWEVD TENGNYDYLM

                                                                               257
            241                                                                300
Am-Lich     YADIDYDHPD VAAEIKRWGT WYANELQLDG FRLDAVKHIK FSFLRDWVNH VREKTGKEMF
Am-Amylo    YADVDYDHPD VVAETKKWGI WYANELSLDG FRIDAAKHIK FSFLRDWVQA VRQATGKEMF
Am-Stearo   YADLDMDHPE VVTELKNWGK WYVNTTNIDG FRLDGLKHIK FSFFPDWLSY VRSQTGKPLF

                                                                               317
            301                                                                360
Am-Lich     TVAEYWQNDL GALENYLNKT NFNHSVFDVP LHYQFHAAST QGGGYDMRKL LNGTVVSKHP
Am-Amylo    TVAEYWQNNA GKLENYLNKT SFNQSVFDVP LHFNLQAASS QGGGYDMRRL LDGTVVSRHP
Am-Stearo   TVGEYWSYDI NKLHNYITKT NGTMSLFDAP LHNKFYTASK SGGAFDMRTL MTNTLMKDQP
```

FIG._5A

```
                                                                                                  377
          361                                                                                     420
Am-Lich   LKSVTFVDNH DTQPGQSLES TVQTWFKPLA YAFILTRESG  YPQVFYGDMY GTKGDSQREI
Am-Amylo  EKAVTFVENH DTQPGQSLES TVQTWFKPLA YAFILTRESG  YPQVFYGDMY GTKGTSPKEI
Am-Stearo TLAVTFVDNH DTNPAKR..CS HGRPWFKPLA YAFILTRQEG  YPCVFYGDYY GI.....PQYNI

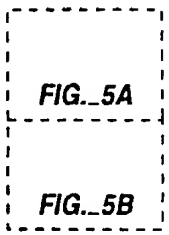
                                                                                                  437
          421                                                                                     480
Am-Lich   PALKHKIEPI  LKARKQYAYG AQHDYFDHHD IVGWTREGDS SVANSGLAAL ITDGPGGAKR
Am-Amylo  PSLKDNIEPI  LKARKEYAYG PQHDYIDHPD VIGWTREGDS SAAKSGLAAL ITDGPGGSKR
Am-Stearo PSLKSKIDPL  LIARRDYAYG TQHDYLDHSD IIGWTREGVT EKPGSGLAAL ITDGAGRSKW

                                                                                  483
          481                                                                                     540
Am-Lich   MYVGRQNAGE TWHDITGNRS EPVVINSEGW GEFHVNGGSV SIYVQR..... .....
Am-Amylo  MYAGLKNAGE TWYDITGNRS DTVKIGSDGW GEFHVNDGSV SIYVQK..... .....
Am-Stearo MYVGKQHAGK VFYDLTGNRS DTVTINSDGW GEFKVNGGSV SVWVPRKTTV STIARPITTR

          541                  559
Am-Lich   ..................... .....
Am-Amylo  ..................... .....
Am-Stearo PWTGEFVRWH EPRLVAWP*
```

FIG._5B

FIG._5



INTERNATIONAL SEARCH REPORT

In ıtional Application No
PCT/US 97/07609

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/56 C12N9/28 C11D9/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L. HOLM ET AL: "Random mutagenesis used to probe the structure and function of Bacillus stearothermophilus alpha-amylase" PROTEIN ENGINEERING, vol. 3, no. 3, 1 January 1990, OXFORD UK, pages 181-191, XP000087332 see Table II (iv) and page 189 Interface between domains A and C ---	1-4,9
A	WO 95 10603 A (NOVONORDISK AS ;BISGAARD FRANTZEN HENRIK (DK); BORCHERT TORBEN VED) 20 April 1995 see page19, line 18 and page 20 line 6 see line 18 --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 1997	15. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 97/07609

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 23873 A (NOVONORDISK AS ;BISGAARD FRANTZEN HENRIK (DK); SVENDSEN ALLAN (DK)) 8 August 1996 see page 19, line 3 - page 20, line 10 ---	1-14
P,X	WO 96 23874 A (NOVONORDISK AS ;SVENDSEN ALLAN (DK); BISGAARD FRANTZEN HENRIK (DK)) 8 August 1996 see page 30 - page 34 -----	1-14

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